In Vivo Antitumor Activity of Anti-CD3-induced Activated Killer Cells

Yeon-Sook Yun, Myrthe E. Hargrove, and Chou-Chik Ting

ABSTRACT

This study investigates the potential of the αCD3-induced killer cells for use in adoptive immunotherapy of tumor growth. The αCD3-induced, activated, killer cells (CD3-AK) were generated in DBA/2 (H-2d) splenocytes by preactivation with αCD3 and were then cultured in the presence (CD3-AK (αCD3+)) or absence (CD3-AK (αCD3−)) of αCD3. The conventional lymphokine-activated killer (LAK) cells were induced by culturing DBA/2 splenocytes with purified human recombinant interleukin 2. Testing their in vitro cytotoxicity against syngeneic mastocytoma P815, CD3-AK (αCD3+) cells gave the highest levels of cytotoxicity and were 20-fold higher than conventional LAK cells and 200-fold higher than CD3-AK (αCD3−) cells. However, the cytotoxic activity of LAK or CD3-AK (αCD3+) cells was augmented by preincubating them with αCD3 for 3 h; then, the difference in cytotoxic activity was reduced from 20- to 4-fold and from 200- to 2-fold, respectively. The in vivo antitumor activity of these killer cells paralleled the in vitro activity. In tests using tumor neutralization experiments, 80–100% of the mice that were challenged with 1 x 10^3 P815 cells remained tumor free after receiving 5 x 10^4 CD3-AK (αCD3+) cells. When the dose challenge increased to 1 x 10^5 and to 1 x 10^6 cells, giving CD3-AK (αCD3+) cells slowed down the rate of tumor growth but only 20% of the mice remained tumor free. The untreated LAK cells or CD3-AK (αCD3−) cells did not induce any protection. After preincubation with αCD3 for 3 h, the CD3-AK (αCD3+) cells provided protection in 30% of the challenged mice. The phenotype of effectors for mediating the in vitro and in vivo antitumor activities was found to be Thy1+, CD4+, and CD8 cells. Flow microfluorometry analysis showed that the higher levels of cytotoxic activity obtained with CD3-AK (αCD3+) cells could not be simply explained by the increase of CD8+ cells, and the cytotoxic activity of individual CD3-AK (αCD3+) cells appeared to be much higher than that of LAK cells. After tumor growth was established for 1–2 days, giving CD3-AK (αCD3+) cells slowed down the rate of tumor growth, and 20% of the mice remained tumor free. These results indicate that CD3-AK cells may be used in the immunotherapy of tumor growth. Major advantages of using CD3-AK cells are their high levels of antitumor activity and their fast growth in vitro; they can be expanded to a 10,000- to 100,000-fold increase in cell numbers in 2–3 weeks. Therefore, it appears that CD3-AK may be an ideal candidate for use in the adoptive immunotherapy of tumor growth.

INTRODUCTION

In the past, repeated attempts to induce specific antitumor immunity in cancer patients have failed. This is largely due to the fact that most "spontaneous" human tumors are nonimmunogenic. Therefore, in recent years attention has been shifted to the induction of nonspecific antitumor immunity. The limited success obtained with combined therapy of LAK cells and IL-2 has provided a promising new modality of cancer therapy (1, 2). However, there are two major shortcomings of this treatment: (a) IL-2 toxicity and (b) the necessity of large numbers of lymphocytes for LAK generation.

There are three major factors that may determine the efficacy of killer cells for adoptive immunotherapy of cancer: (a) selective cytotoxicity against tumor targets, (b) high levels of antitumor cytotoxicity, and (c) generation of sufficient numbers of killer cells in a reasonably short period of time. We found that antibody against CD3 of the T-cell receptor complex elicited the generation of killer cells through the activation of endogenous lymphokine production (3). Similar to LAK cells, CD3-AK cells selectively killed tumor targets. These CD3-AK cells gave 10- to 50-fold higher cytotoxic activity than the conventional LAK cells. In addition, the CD3-AK cells could be maintained in vitro with active growth for at least 4–5 weeks, whereas the conventional LAK cells usually ceased to grow after 7–10 days in culture. Therefore, the CD3-AK cells may provide an alternative choice for use in the immunotherapy of cancer. In this communication, we examined the in vivo antitumor activity of CD3-AK cells and compared it with the antitumor activity of LAK cells.

MATERIALS AND METHODS

Mice. Female DBA/2J and C57BL/6 mice, age 2–5 months, were obtained from the Veterinary Research Branch, Division of Resources Services, NIH (Bethesda, MD), and from The Jackson Laboratory (Bar Harbor, ME).

HrIL-2. Purified human recombinant IL-2 was kindly provided by the Cetus Corporation (Emeryville, CA).

Monoclonal Antibodies. Four monoclonal antibodies were produced in the following hybridoma cell lines: (a) αCD3 from hybridoma line 145-2C11 (4), (b) αThy1.2 from hybridoma line HO-13-4 (5), (c) αCD4 from hybridoma line GKL.5 (6), and (d) αCD8 from hybridoma line 83-12-5 produced by Dr. Jeffrey Bluestone (University of Chicago, Chicago, IL). All were culture supernatants.

Generation of CD3-AK Cells. The culture medium was RPMI 1640 that contained 5% fetal bovine serum (GIBCO, Grand Island, NY), 2 x 10^{-2} M N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid buffer, 5 x 10^{-3} M 2-mercaptoethanol, and 50 μg/ml gentamicin. The CD3-AK cells were generated by preactivation of normal DBA/2 spleen cells with a 1:10 dilution of αCD3. After 1 day, a portion of the cells were cultured with a 1:30 dilution of αCD3 and supplemented with 30 units/ml HrIL-2, and these cells were designated as CD3-AK (αCD3+). Other cells were washed twice and resuspended in fresh culture medium supplemented with 30 units/ml HrIL-2, and these cells were designated as CD3-AK (αCD3−).

Generation of LAK Cells. LAK cells were generated by culturing normal DBA/2 spleen cells with a high dose (1000 units/ml) or a low dose (50 units/ml) of HrIL-2 (7).

Target Cells. Two tumor lines were used: mastocytoma P815 of H-2b haplotype (8), and Friend virus-induced leukemia HFL/b of H-2b haplotype (9). Con A-induced T-lymphoblasts were induced by culturing DBA/2 (H-2b) or C57BL/6 (H-2b) spleen cells with 2.5 μg/ml Con A for 2 days and were then grown in IL-2-supplemented medium for an additional 2–3 days. The LPS-induced B-lymphoblasts were induced with 20 μg/ml LPS.

Cell-mediated Cytotoxicity Assays. A 20-h 125I-UdR release assay was used in most experiments. Details of the technique have been described elsewhere (10). The results are expressed as total percentage of lysis and net percentage of lysis, as previously described (3, 10). One lytic unit was defined as the number of effector cells to give 30% lysis (net percentage of lysis).

51Cr Release Assay. The 51Cr-labeled targets were used in a 4–6-h incubation assay. The procedures were similar to those described above.

In Vivo Tumor Transplantation Experiments. (a) In the Winn assay, the tumor neutralization experiments were performed by i.p. inocula-
tion of 5 x 10^6 effector cells and graded doses of P815 tumor cells. The development of ascites growth and mortality rate were observed for 50 days. (b) Adoptive immunotherapy was performed by i.p. inoculation of 1 x 10^7 P815 cells 0-3 days prior to the inoculation of 5 x 10^6 CD3-AK cells. In the in vivo experiments, three to five mice were used in each group. Some experiments were repeated more than 3 times and the results were reproducible.

FMF. The expression of CD3, CD4, or CD8 on the cultured CD3-AK or LAK cells was determined by dual laser FMF. Fluorescein isothiocyanate-conjugated antibodies against CD3, CD4, or CD8 were used to detect these markers, and fluorescein isothiocyanate-α-Leu 4 was used as control.

RESULTS

Cytotoxic Activity of CD3-AK Cells and LAK Cells. The CD3-AK cells were induced by 1-day activation of DBA/2 (H-2^d) spleen cells with αCD3 and then they were cultured in the presence [CD3-AK (αCD3^)] or absence [CD3-AK (αCD3^-)] of αCD3. The LAK cells were induced with a high dose (1000 units/ml) or a low dose (50 units/ml) of hrIL-2. These activated killer cells were tested on day 5 by a 20-h incubation 125I-UdR release assay against tumor targets of syngeneic (P815) or allogeneic (HFL/b) origin, and against syngeneic or allogeneic Con A-induced T-lymphoblasts (Fig. 1). The CD3-AK cells selectively killed tumor targets (P815 and HFL/b) and did not kill Con A-induced lymphoblasts of either syngeneic or allogeneic origins. The conventional LAK cells killed both tumor targets. In addition, LAK cells induced with the high dose of IL-2 also killed lymphoblasts of both syngeneic and allogeneic origins. The LAK cells induced with the low dose of IL-2 did not kill lymphoblasts.

In comparing the cytotoxic activity of CD3-AK and LAK cells against syngeneic tumor P815 and T- or B-lymphoblasts (Fig. 2), we found that, in a short term (4 h) ^51Cr release assay, CD3-AK (αCD3^-) did not give significant cytotoxicity against P815, but LAK cells gave low levels of cytotoxicity. High levels of cytotoxicity were obtained with CD3-AK (αCD3^) cells. However, in a 20-h 125I-UdR release assay, low (4 LU) to moderate (40 LU) levels of cytotoxicity against tumor target P815 were obtained with CD3-AK (αCD3^-) or LAK cells, respectively, and high levels (800 LU) of cytotoxicity were obtained with CD3-AK (αCD3^) cells, and there was a 20- to 200-fold difference in cytotoxic activity among these killer cells. These effectors did not kill Con A-induced T-lymphoblasts, but the CD3-AK (αCD3^) cells killed LPS-induced B-lymphoblasts mediated by αCD3-redirected killing through Fc receptors (11). After incubation with αCD3 for 3 h, the cytotoxic activities of all these effectors were augmented. The augmented CD3-AK (αCD3^) killing (400 LU) was higher than the augmented LAK killing (200 LU), and these levels were only 2- to 4-fold less than the untreated CD3-AK (αCD3^-) killing (800 LU).

In Vivo Antitumor Activity. In testing the in vivo antitumor activity of the CD3-AK and LAK cells (Fig. 3), we found that complete protection was obtained by giving 5 x 10^6 CD3-AK (αCD3^) cells. Giving the same numbers of normal spleen, LAK, or CD3-AK (αCD3^-) cells offered no protection. After preincubation with αCD3 for 3 h, the treated CD3-AK (αCD3^) cells provided 30% protection in P815-challenged mice; the complete protection was obtained by giving 5 x 10^7 CD3-AK (αCD3^-) cells. Giving the same numbers of normal spleen, LAK, or CD3-AK (αCD3^-) cells, with or without preincubation 3 h with a 1:10 dilution of αCD3, the untreated CD3-AK (αCD3^-) cells killed LPS-induced B-lymphoblasts selectively, and these levels were only 2- to 4-fold less than the untreated CD3-AK (αCD3^-) killing (800 LU).
The protective effect of CD3-AK (<CD3*) cells was dose dependent (Fig. 6). Complete or near complete protection (80-100%) was achieved by giving 5 x 10⁶ CD3-AK (αCD3*) cells, when challenged with 1 x 10⁶ P815 cells. When challenged with 10- to 100-fold more tumor cells (1 x 10⁵ and 1 x 10⁴ P815 cells), the CD3-AK (αCD3*) cells slowed down the rate of the tumor growth, and all mice eventually died of progressive tumor growth at a later time. There was no protective effect on tumor growth when the challenge dose reached 1 x 10⁴ cells.

These results clearly showed that CD3-AK cells were much more effective than the LAK cells in inhibiting the growth of P815 cells.

**Table 1 In vivo antitumor activity of CD3-AK and LAK cells**

<table>
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<tr>
<th>Donor effectors&lt;sup&gt;α&lt;/sup&gt;</th>
<th>Tumor take&lt;sup&gt;β&lt;/sup&gt;</th>
<th>P&lt;sup&gt;α&lt;/sup&gt;</th>
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<tr>
<td>Experiment 1</td>
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<tr>
<td>Normal spleen</td>
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<td>LAK</td>
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<sup>α</sup> DBA/2 mice were challenged with 1 x 10⁴ P815 cells i.p. and were simultaneously treated with 5 x 10⁶ normal splenocytes, CD3-AK (αCD3*), CD3-AK (αCD8*), or LAK cells, as indicated. The development of ascites tumor growth was observed for 45-50 days. The mice that developed tumor growth usually died around days 25-30.

<sup>β</sup> Numbers of mice that developed tumor growth per total numbers of mice that were challenged with P815 cells.

<sup>α</sup> Determined by the χ² test.

**Fig. 4.** Phenotype of the CD3-AK effectors in cytotoxicity assay. The CD3-AK (αCD3*) cells were depleted of Thy1*, CD8*, or CD4* cells, and then their residual cytotoxicity was tested against P815 in a 3H-UdR release assay at indicated effector:target ratios. C’, complement.

**Fig. 5.** Phenotype of the CD3-AK effectors responsible for in vivo antitumor activity. The CD3-AK (αCD3*) cells were depleted of Thy1*, CD8*, or CD4* cells, and then they were tested in tumor neutralization experiments to determine their protective effect on the challenge of P815 cells. Development of tumor growth and survival rate were observed for 50 days. All mice that were challenged with 1 x 10⁶ P815 cells alone died on approximately day 30. When comparing the tumor incidence with the control group (5 of 5), P < 0.04 for the group which received untreated CD3-AK cells, P < 0.05 for the group which received complement (C')-treated or CD4-depleted CD3-AK cells. For all other groups, P > 0.05. When the statistical evaluation was done on day 20, P < 0.05 for the groups which were challenged with 1 x 10⁴ and 1 x 10⁵ P815 cells. Ø, P815 alone, 1 x 10⁶; ＃, P815 1 x 10⁴ + CD3-AK (αCD3*); Δ, CD3-AK (αCD3*)-Thy-; △, CD3-AK (αCD3*)-CD8-; ◆, CD3-AK (αCD3*)-CD4-; ●, CD3-AK (αCD3*)-C’.

**Fig. 6.** Effect of the size of tumor inoculum on the in vivo protection by CD3-AK cells. DBA/2 mice were treated i.p. with 1 x 10⁵ to 1 x 10⁶ P815 cells plus 5 x 10⁶ CD3-AK (αCD3*) cells. Development of tumor growth and survival rate were observed for 50 days. When comparing the tumor incidence with the control group (5 of 5), P < 0.03 for the group which received 1 x 10⁵ P815 cells and CD3-AK cells. For all other groups, P > 0.05. When the statistical evaluation was done on day 20, P < 0.05 for the groups which were challenged with 1 x 10⁴ and 1 x 10⁵ P815 cells. Ø, P815 alone, 1 x 10⁶; ＃, P815 1 x 10⁴ + CD3-AK (αCD3*); Δ, P815 1 x 10⁴ + CD3-AK (αCD3*); ◆, P815 1 x 10⁴ + CD3-AK (αCD3*)-Thy-; ●, P815 1 x 10⁴ + CD3-AK (αCD3*)-CD8-; △, CD3-AK (αCD3*)-CD4-.

**Fig. 7.** Adoptive Immunity. To test whether long lasting immunity was induced in mice receiving CD3-AK (αCD3*) cells and then challenged with P815 cells, these mice were rechallenged with P815 cells (Fig. 7). We found no protection in these mice.

**Distribution of Subsets of T-Cells after Activation by αCD3 or IL-2.** It has been shown in our previous reports that, after activation by IL-2, both CD8* and CD8* LAK cells were induced (7). The CD8* LAK cells were responsible for mediating killing against P815 cells, and the CD3-AK cells which killed the P815 cells were also exclusively CD8* cells (3). To determine the phenotype of the subsets of T-cells present in the LAK cells and CD3-AK cells, 1-week cultured cells were analyzed by FMF. The results are summarized in Table 2. It shows that the numbers of CD8* cells increased with increasing doses of IL-2, whereas the numbers of CD3* cells decreased with increasing doses of IL-2. The CD4* cells remained as a small percentage (2-4%). There were over 80% CD8* cells in CD3-AK cells, less than 2-fold higher than the LAK cells which were induced with 300-1000 units/ml IL-2.

**Transfer of CD3-AK Cells Did Not Provide Long Lasting Adoptive Immunity.** To test whether long lasting immunity was induced in mice receiving CD3-AK (αCD3*) cells and challenged with P815 cells, these mice were rechallenged with P815 cells (Fig. 7). We found no protection in these mice.

**Adaptive Immunotherapy with CD3-AK Cells.** To test whether CD3-AK cells could truly provide therapeutic effect, the mice
AK (αCD3+) cells, there was a significant delay of tumor growth when mice were challenged 1 to 2 days before being given CD3-AK (αCD3−) cells (Fig. 8), and the protective effect of CD3-AK (αCD3+) cells was then evaluated. It was found that, when mice were challenged 1 to 2 days before being given CD3-AK (αCD3+) cells, there was a significant delay of tumor growth and 20% of the mice remained tumor-free. No protection was seen when the mice were challenged 3 days before being given CD3-AK (αCD3+) cells.

**DISCUSSION**

The initial success achieved by treating cancer patients with LAK cells and IL-2 (1) has revived the interest in immunotherapy of cancer. In addition to LAK cells, tumor-infiltrating lymphocytes have also been used in some cancer patients (12, 13). The NK cells are another cell type that has been considered for use in the treatment of cancer patients (14). In the aforementioned three cell types (LAK, tumor-infiltrating lymphocytes, and NK), one limiting factor is obtaining sufficient amounts of effector cells. We showed that αCD3-induced killer cells were highly cytotoxic for tumor targets and could be quickly expanded in culture due to their fast growth rate (3).

Both CD3-AK and LAK cells showed selective cytotoxic activity against tumor cells (3, 15, 16). Recently, it has been shown that LAK cells also killed allogeneic lymphoblasts (17). However, we found that this phenomenon was only seen with LAK cells generated with a high dose (1000 units/ml) of IL-2 (Fig. 1), and LAK cells induced with lower doses of IL-2 (less than 300 units/ml) did not kill Con A-induced T-cell blasts (data not shown). There was no discrimination between syngeneic or allogeneic Con A-induced T-lymphoblasts. Therefore, LAK cells generated with a high dose of IL-2 may cause damage to some normal tissues. On the other hand, CD3-AK (αCD3+) cells were maintained in lower doses of IL-2 (30–50 units/ml) and, thus, they did not kill T-lymphoblasts but did kill LPS-induced B-lymphoblasts, mediated through αCD3-directed killing against Fc receptor-bearing cells (11). Therefore, CD3-AK (αCD3+) cells may cause damage to Fc receptor-bearing cells.

For the susceptible targets, the cytotoxic activity of CD3-AK (αCD3+) cells was usually 10–50 times higher than that of LAK cells (e.g., P815; Figs. 1 and 2). After 1–2 weeks of culturing, the cytotoxic activity of CD3-AK (αCD3+) cells reached the same level as that of the CD3-AK (αCD3+) cells.

We previously reported that αCD3 augmented the cytotoxic activity of LAK cells, and the reactions were mediated by CD8+ cells (7). In the present study, it was also shown that, after LAK cells were preincubated for 3 h with αCD3, cytotoxic activity of these LAK cells against P815 increased 5-fold (from 40 LU to 200 LU) (Fig. 2). A more striking increase was seen with CD3-AK (αCD3−) cells after a 3-h incubation with αCD3 (from 4 to 400 LU, a 100-fold increase) (Fig. 2). After incubation with αCD3, there was only a 2- or 4-fold difference between untreated CD3-AK (αCD3−) and CD3-AK (αCD3+) or LAK cells, respectively. Prior to the treatment, there was a 200- or 20-fold difference between them (Fig. 2).

The in vivo antitumor activity of CD3-AK and LAK cells against P815, tested in tumor neutralization experiments, paralleled the in vitro cytotoxic activity of these killer cells (Fig. 3; Table 1). Only CD3-AK (αCD3+) cells provided complete protection against the challenge of P815 cells. Normal spleen, LAK, or CD3-AK (αCD3+) cells failed to give any protection. After preincubation with αCD3 for 3 h, only CD3-AK (αCD3+) cells gave partial protection.

There was a significant delay of tumor growth and 20% of the mice remained tumor-free. No protection was seen when the mice were challenged 3 days before being given CD3-AK (αCD3+) cells.

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**Table 2 Distribution of subsets of T-cells after activation by IL-2 or αCD3**

| Lymphocytes | IL-2 (units/ml) | Experiment 1 | | Experiment 2 | |
|-------------|----------------|-------------|--------------------------|--------------------------|
|             |                 | CD4+ | CD8+ | CD3+ | CD8+ | |
| Normal spleen | 50 | 9 | 31 | 84 | 32 |
| LAK          | 100 | 3 | 31 | 82 | 47 |
| LAK          | 1000 | 3 | 52 | 39 | 72 |
| CD3-AK (αCD3+) | 50 | 19 | 81 | 92 | 86 |
| CD3-AK (αCD3+) | 50 | 11 | 72 | 2 | 82 |

*a The LAK cells were generated with different doses of IL-2 as indicated. The CD3-AK cells were maintained in 50 units/ml IL-2 with or without αCD3.

*b The LAK cells or CD3-AK cells were cultured for 1 week before testing.

The percentage of CD3+, CD4+, or CD8+ cells was determined by FMF, as described in "Materials and Methods."
increase of CD8+ cells in the former group (Table 2); there was a less than 2-fold difference in the numbers of CD8+ cells and yet there was 10-50-fold higher cytotoxic activity in CD3-AK cells. Therefore, it appeared that the cytotoxic activity of individual CD3-AK cells was much higher than that of the LAK cells.

The protective effect of CD3-AK (αCD3+) cells was dose dependent (Figs. 3, 5, 6, and 8). It appears that the size of the tumor burden affects the efficacy of the CD3-AK cells on tumor growth. In addition, no active immunity was induced after adoptive therapy with CD3-AK cells (Fig. 7). All mice that were rechallenged with P815 showed the same rate of tumor progression as the untreated mice. Therefore, no specific antitumor immunity was induced in these treatments, indicating that P815 cells were nonimmunogenic.

In exploring the possibility of using CD3-AK cells in immunotherapy, we found that, when CD3-AK cells were given after tumor growth was established, they could slow down the rate of tumor progression and 20% of the mice remained tumor-free when P815 cells were given 1 to 2 days prior to giving CD3-AK (αCD3+) cells. In these experiments, only 5 × 10^6 CD3-AK cells were given. If more effector cells were given, better results might have been achieved. Nevertheless, the data suggest that CD3-AK cells could be used in adoptive immunotherapy of tumor growth.

Similar to the adherent population of LAK cells (14), the CD3-AK cells could be maintained in culture for a prolonged period of time. One major advantage of using CD3-AK cells is their fast growth in vitro. In 8 days, they can be expanded from 1 × 10^7 cells to 1 × 10^10 cells (Fig. 9), whereas the LAK cells need a much longer period of time. One major advantage of using CD3-AK cells is their fast growth in vitro. In 8 days, they can be expanded from 1 × 10^7 cells to 1 × 10^10 cells (Fig. 9), whereas the LAK cells require much smaller amounts of tumor progression and 20% of the mice remained tumor-free when P815 cells were given 1 to 2 days prior to giving CD3-AK (αCD3+) cells. In these experiments, only 5 × 10^6 CD3-AK cells were given. If more effector cells were given, better results might have been achieved. Nevertheless, the data suggest that CD3-AK cells could be used in adoptive immunotherapy of tumor growth.

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It appears that, for susceptible tumors, the CD3-AK cells have several advantages over LAK cells. The CD3-AK cells give higher levels of cytotoxic activity, both in vitro and in vivo. They can be expanded in vitro in large numbers in a relatively short period of time, and they require much smaller amounts of IL-2 for expansion. It is possible that, if IL-2 is required for combined therapy with CD3-AK, a much lower dose may achieve the purpose and thus reduce the IL-2-induced toxicity. Therefore, CD3-AK cells may be an ideal candidate for use in adoptive immunotherapy of tumor growth that is susceptible to killing by these activated killer cells.

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